Review

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Can the silkworm (*Bombyx mori*) be used as a human disease model?

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Summary ***Bombyx mori* (silkworm) is the most famous lepidopteran in Japan. *B. mori* has long been used in the silk industry and also as a model insect for agricultural research. In recent years, *B. mori* has attracted interest in its potential for use in pathological analysis of model animals. For example, the human macular carotenoid transporter was discovered using information of *B. mori* carotenoid transporter derived from yellow-cocoon strain. The *B. mori* carotenoid transport system is useful in human studies. To develop a human disease model, we characterized the human homologs of *B. mori*, and by constructing KAIKO functional annotation pipeline, and to analyze gene expression profile of a unique *B. mori* mutant strain using microarray analysis. As a result, we identified a novel molecular network involved in Parkinson's disease. Here we describe the potential use of a spontaneous mutant silkworm strain as a human disease model. We also summarize recent progress in the application of genomic information for annotation of human homologs in *B. mori*. The *B. mori* mutant will provide a clue to pathological mechanisms, and the findings will be helpful for the development of therapies and for medical drug discovery.**

Keywords: Silkworm, human disease model, *Bombyx mori* mutant, Parkinson's disease, translucent larval skin

1. Introduction

The silkworm (*Bombyx mori*), which produces silk fiber in its silk glands, is the most famous lepidopteran in Japan. It was domesticated by humans more than 5,000 years ago in China. *B. mori* cannot survive without human help.

In the 19th century, silkworm was an important livestock animal in Japan. Nature and Science Museum of Tokyo University of Agriculture and Technology (*http://www.tuat.ac.jp/~museum/*) displays several ukiyo-e that depicts scenes of sericulture at that time. One ukiyo-e depicts women reeling cocoons (Figure 1A), and another depicts women rearing silkworms (Figure 1B). Thus, rearing silkworms, reeling cocoons and weaving cloth were done by the women. These methods of sericulture were recorded in some books. Silkworms were important possessions for the Japanese people. They preferred colored to white cocoons, and variety of colored cocoons were produced by Japanese sericultureists till the 19th century. However, it has not been clear why whited cocoon strains were standardized at the early 20th century. The Japanese people collected many silkworm strains from the Europe and China. Because Japanese sericultureists were seeking for silkworm strains which were disease-resistant and made the big cocoon. In this process, Japanese sericultureists obtained various phenotypes, and discovered many unique mutants. Therefore, Japanese sericultureists have maintained these many silkworm races of these for the future gene resource need.

Additionally, Japanese geneticist's Toyama rediscovered the Mendel's law of heredity in animal using white and yellow cocoon race of *B. mori* in 1906 (*1*). Thereby, the phenotypes of *B. mori* were genetically linked to the causative genes on the linkage maps of *B. mori*. Currently, 456 mutant strains are maintained at the National Bio Resource Project (NBRP) KAIKO (*http://*

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Figure 1. Sericulture and silkworm depiction on Ukiyoe.

silkworm.nbrp.jp/) located at Kyushu University.

The silkworm has been used as a model insect for agricultural research for several reasons: *i*) the majority of agricultural pests are lepidopterans, *ii*) its genome sequence is well characterized, *iii*) various genetic mutants are available, and *iv*) silkworm is amenable to transgenic, knock-out, and microarray technologies (*2-7*). However, to date, silkworms have never been used as human disease models.

Here we describe the potential use of a spontaneous silkworm mutant strain as a human disease model, based on our current study (*8*). We also summarize recent progress in the application of genomic information for the annotation of human homologs in *B. mori*. These findings will help elucidate molecular mechanisms of human diseases.

2. The human macular carotenoid transporter discovered using an information of *B. mori*

There are several known colored cocoons: yellow, pink, golden yellow, straw, green, and sasa (yellowish green). Yellow-, pink-, golden-yellow-, and flesh-colored cocoon pigments are derivatives of carotenoids (*9*), whereas sasa- and green-cocoon pigments are derivatives of flavonoids (*10,11*). These pigments protect pupae from sunlight as antioxidants (*12*). A carotenoid-binding protein (CBP) was identified in a *B. mori* yellow-cocoon strain in 2002 (*13*) as an intracellular carotenoid transporter in the silk gland by a biochemical approach. Interestingly, CBP was categorized as the first member of a steroidogenic acute regulatory protein (StAR) family whose members bind only carotenoids in their lipid-binding domains.

Landrum *et al*. (*14*) have reported that carotenoids are accumulated in the human macula as macular pigment. A low concentration of macular pigment increases the risk of age-related macular degeneration from blue light damage. However, sustained intake of dietary carotenoids may reduce the risk of age-related human macular degeneration (*14*). Thus, identification of a macular carotenoid transporter is important for elucidation of the molecular mechanism of macular degeneration.

The antibody to *B. mori* CBP has cross-reactivity to macular carotenoid transporter identified from human retina and layers of the primate macula where the macular carotenoid pigment is at its highest concentration (*15*). The human macular carotenoid transporter StARD3 (also known as MLN64) was discovered by Bernstein *et al*. in 2009 (*16*).

The *B. mori* CBP information and antibody were helpful for identification of the human macular carotenoid transporter. Therefore, the common molecular mechanisms were present in the carotenoid transport system between *B. mori* and human.

In the present study, a *B. mori* cocoon-color mutant was helpful for elucidation of the molecular mechanism of age-related human macular degeneration (*17*).

3. How many human orthologs does silkworm have?

A draft silkworm genome sequence was completed by Chinese and Japanese groups in 2004 (*2,3*). In 2013, Suetsugu *et al*. (*4*) reported that the silkworm genome contained 16,823 gene loci, based on sequence analysis of cDNA data set. However, these data are not included in public databases currently. We accordingly identified human homologs using the Ensembl Metazoa (*18*) predicted-protein data set of *B. mori*.

There are challenges in using silkworm genome information. Silkworm genome sequences have been analyzed, but the functional annotation of the genes still remains obscure. If we can annotate the *B. mori* genome in the same manner as the *Drosophila melanogaster* genome, we can use the public databases to mammalian model organisms and commercial pathway softwares for microarray analysis or next-generation sequencing (NGS) data. Finally, we can analyze these big data from microarray or NGS analysis as deeply as for mammalian model organisms. *D. melanogaster* has been used as a human disease model in studies involving gene-gene interactions. A systematic BLAST search (*19*) revealed 548 human disease-associated genes in *D. melanogaster*. Importantly, *D. melanogaster* is the only insect species in which gene annotations have been extensively assigned (*20,21*), and many human disease models have been developed. However, the analysis of human disease-associated genes was reported by Reiter *et al*. (*19*) in 2001 and the complete human genome was not available at that time. Thus, we needed the re-analysis of updated human gene set in the current study. To annotate silkworm genome information, we identified human homologs common to *B. mori* and *D. melanogaster*; we obtained the cDNA sequence sets of these species from Ensembl Metazoa, and we performed systematic BLAST search to identify human homologs in these species. *B. mori* contained 8,469

Table 1. Comparison of human homologs between *Bombyx mori* and *Drosophila melanogaster*

Species	Human homolog transcripts	Total transcripts	Human homolog genes	Total (genes)	Ratio (%) of human homolog/total genes
B. mori	8469	14623	8469	14623	58
D. melanogaster	21230	30362	8815	13918	63

Table 2. Gene enrichment analysis of human homolog genes in *Bombyx mori*

KEGG ID	KEGG pathway	Count	*p* value
hsa05016	Huntington's disease	82	2.1E-4
hsa05010	Alzheimer's disease	68	1.1E-2
hsa05012	Parkinson's disease	51	1.1E-2
hsa04120	Ubiquitin mediated proteolysis	67	7.0E-5
hsa00190	Oxidative phosphorylation	54	2.5E-2
hsa03050	Proteasome	31	8.7E-6

4,020 genes shared with 57 Kyoto Encyclopedia of Genes and Genomes pathways.

and *D. melanogaster* contained 8,815 human homologs (Table 1). Thus, *B. mori* had 58% of the human homologs in the genes. Furthermore, we characterized these human homologs in *B. mori* by enrichment analysis using the DAVID bioinformatics database (*https://david.ncifcrf.gov/home.jsp*). The human homologs were included in 57 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and the genes in the most conserved pathways in the DAVID analysis were concerned in neurodegenerative disease, oxidative stress, and protein degradation-associated genes (Table 2). In addition, these pathways were also conserved in *D. melanogaster*, and corresponding related human disease models were developed. The FlyBase Human Disease Model Report List (*http://flybase.org/static_pages/FBhh/browse.html*) contains 59 human disease models in *D. melanogaster*. The basic information of human disease related genes were obtained from Online Mendelian Inheritance in Man database. Human disease related fly ortholog was modified as functional disturbance, and the genetically modified fly was created. These human disease model flies are reported in the public database and stock centers. Accordingly, *B. mori* may have potential use as a human disease model similar to *D. melanogaster*.

4. Abundance of spontaneous silkworm mutant stocks in Japan

The NBRP KAIKO also maintains 456 spontaneous mutant *B. mori* strains. These genes responsible for each mutation have been mapped in the *B. mori* linkage map information. User can choose the mutant which correspond to gene symbols and mutant strains on the NBRP web site (*http://www.shigen.nig.ac.jp/silkwormbase/ViewCausativeGene.do?*). Some causative genes of the product have been discovered and are shown in a table on the NBRP web site. However, many causative genes in *B. mori* mutants have not been identified. Importantly, spontaneous mutants have unique phenotypes that do not appear in other model species. For instance, there are a variety of cocoon colors, egg colors, and larval skin colors and patterns; translucent larval skin; and black-colored adults. If we can identify the causal genes responsible for these phenotypes as human homolog, the discovery will contribute to identify novel molecular mechanisms that could not be detected using other model organisms.

Thus, *B. mori* has many valuable spontaneous mutants in Japan. We propose that *B. mori* is a good human disease model candidate, on the basis of these spontaneous mutants.

5. KAIKO functional annotation pipeline is useful for screening target molecules

B. mori has 26 mutants in uric acid metabolism. The common phenotype of these mutants is translucent larval skin (*22*). Eight genes have been identified as causative genes in translucent larval skin mutants (*23-31*). These genes are involved in the synthesis or uptake of uric acid.

To analyze the *B. mori* translucent larval skin mutant strain o751 (*op*), we constructed KAIKO functional annotation pipeline using corresponding information of *B. mori* human homologs and human genes (Figure 2).

The *B. mori op* mutant is classed as a translucent larval skin mutant (Japanese name, aburako) and displays occasional unique actions such as vibration. Classical linkage analysis has shown that the *op* gene is located on chromosome 23, and involved in the phenotypes of the extraordinarily high mortality, particularly in the pupal stage, and the male infertility except for the oily mutation (NBRP silkworm database; *http://www.shigen.nig.ac.jp/silkwormbase/ViewStrainDetail.do?id=309*). We investigated gene expression in the *B. mori op* mutant using microarray analysis with KAIKO functional annotation pipeline. We identified a novel uric acid synthesis-modulated pathway (Figure 2, gray-colored molecule) (*8*).

6. Parkinson's disease and uric acid

Parkinson's disease (PD) remains an incurable disease. Its mechanisms responsible for dopaminergic neuronal cell degradation cause oxidative stress or protein accumulation by ubiquitin proteasome failure, and this damage depletes dopamine levels in substantia

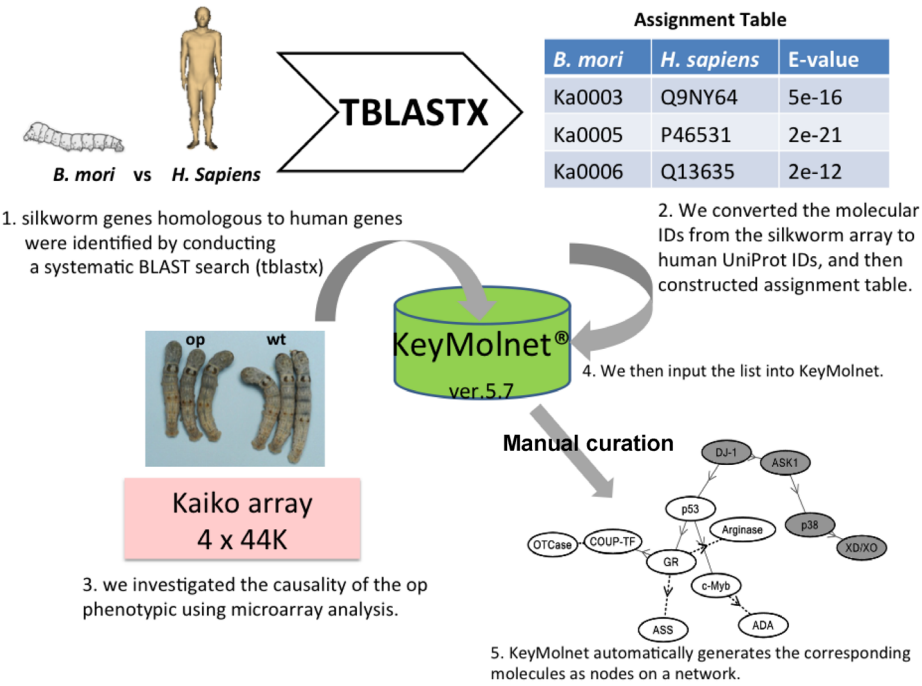

Figure 2. Screening of target molecule using KAIKO functional annotation pipeline.

nigra neurons (*32,33*). Genetic studies have identified 21 genes associated with PD at different loci based on family linkage analysis [PD; Online Mendelian Inheritance in Man 168600]. PD-associated gene knockout animal models have been developed as familial PD models (*34*).

The majority of sporadic PD onset is caused by environmental stress (*35,36*), and a molecular mechanism of oxidative stress has been developed. In animal models of sporadic PD, oxidative stress has been simulated using mitochondrial complex I inhibitors (*37*).

The final product of purine metabolism, uric acid, plays an important role as a physiological antioxidant (*38*). Several groups have reported a correlation between decreased plasma uric acid concentrations and clinical progression and stage of PD (*39-45*). Conversely, high plasma uric acid concentrations in hyperuricemia may reduce the risk and delay the progression of PD, but it increases the risk of cardiac diseases (*46*). Thus, uric acid has a dual function in organisms. In the case of PD, uric acid may be expended to resist oxidative injury (*47*); however, the molecular mechanism underlying the decrease in plasma uric acid concentration in advanced clinical stages of PD has not been analyzed using either of these model animals. Owing to the lack of adequate animal models, the function of uric acid in PD pathogenesis is poorly understood.

7. Why is a silkworm model a good candidate for the analysis of Parkinson's disease and uric acid?

It is well established that the uric acid metabolism process uses *B. mori* mutants. Uric acid is synthesized mainly in the fat body and is thereafter transported to the integument via the hemolymph. It is the end product of purine degradation *via* xanthine/hypoxanthine reactions catalyzed by xanthine dehydrogenase. It is eliminated through the Malpighian tubules. Uric acid accumulates as urate granules and produces a whitening of the integument. In translucent larval skin mutants, it shows abnormal accumulation in the integument (*22*).

Uric acid plays a protective role against photooxidative stress in *B. mori*, as shown by a markedly reduced survival rate in larvae under UV irradiation with injection of allopurinol, an inhibitor of uric acid synthesis (*48*). It directly scavenges oxygen radicals and may play an important role in protection against environmental oxidative stress in *B. mori*.

Only *B. mori* translucent larval skin mutants show abnormality in integument color in the larval developmental stage. Other model organisms do not show a similar phenotype.

In human studies, plasma uric acid concentrations decrease following the clinical progression and stage development of PD (*39-45*). However, molecular mechanisms underlying reduction in plasma uric acid concentrations remain unknown. Reduced plasma uric acid concentrations are due to consumption of uric acid as an antioxidant in PD. Furthermore, the causative gene of human PD induces strong oxidative stress in the central nervous system (*47*). Moreover, the regulation of xanthine dehydrogenase phosphorylation in the uric acid synthesis pathway is unclear.

We accordingly investigated gene expression in the *B. mori op* mutant using KAIKO functional annotation pipeline for analysis of microarray data. We identified

a novel uric acid synthesis-modulating pathway (Figure 2). We speculated that these molecules relate to the phosphorylation of the protein (*8*). However, we were unable to identify the *op* causative gene in the present study.

Molecular mechanisms associating decreased plasma uric acid concentrations with PD remain obscure. *B. mori* translucent larval skin mutants provide promising clues for elucidation of these mechanisms and for development of therapies and drugs for PD. Further study of genes with common function in the uric acid synthesis pathways of humans and *B. mori* is warranted.

Do you now believe that silkworms can be used as human disease models?

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